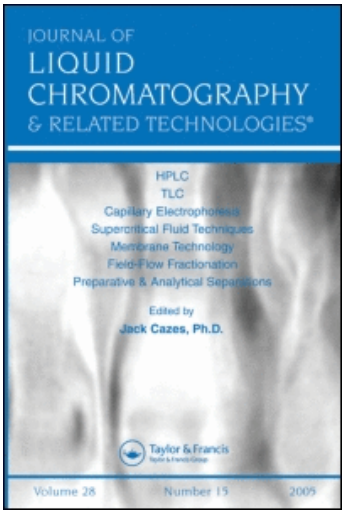

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Supercritical Fluid Extraction of Microcystin LR from Cyanobacteria using Aqueous Acetonitrile Modifier

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Abstract: The use of supercritical fluids for the analytical extraction of the nonvolatile, higher molecular weight compound, microcystin LR, from cyanobacteria was investigated. The microcystin LR included in this study was sparingly soluble in neat supercritical fluid CO_2. However, the microcystin LR was successfully extracted with a ternary mixture (90% CO_2, 9.5% acetonitrile, 0.5% water) at 40°C and 250 atm. The polar carbon dioxide-aqueous acetonitrile fluid system gave a high extraction efficiency for the extraction of the polar microcystin LR from cyanobacteria. In our laboratory, in the past, aqueous acetic acid-modified CO_2 was used for the extraction of microcystins from cyanobacteria and aqueous methanol was also used as a modifier. In this study, we tried a new organic solvent, aqueous acetonitrile as a co-solvent, so that we could obtain a higher extraction efficiency of the microcystin LR from cyanobacteria than in earlier experiments.

Keywords: Supercritical fluid extraction, Microcystin LR, Cyanobacteria, Acetonitrile

INTRODUCTION

Algal blooms, in particular cyanobacterial blooms, are a major issue for water authorities, causing significant taste and odour problems. The knowledge that many of these blooms are toxic has changed the concern from a purely aesthetic problem to one that affects human health. Over 20 different cyclic peptide hepatotoxins, termed microcystins,[1] have been isolated from cyanobacteria (blue-green algae).[2,3] While *Microcystis* is the most studied genus, species

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in the genera *Anabaena, Nodularia, Nostoc*, and *Oscillatoria* also contain these toxins.[4–6] Microcystins (Figure 1) are characterized as monocyclic heptapeptides containing a common moiety comprising 3-amino-9-methoxy-10-phenyl-2,3,8-trimethyldeca-4,6-dienoic acid (Adda), N-methyldehydroalanine (Mdha), D-alanine, β-linked D-erythro-β-methylaspartic acid and $\gamma-$linked D-glutamic acid, plus two L-amino acids as variants.[7,8]

Toxic cyanobacterial blooms have been reported in many countries.[9] Toxic waterblooms cause death of domestic animals and wildlife, and human illness. Cyanobacterial toxins are toxic to zooplankton and fish[10] and can be accumulated in fish and aquatic animals.[11] The structures and toxic functions are classified into three groups: neurotoxin, hepatotoxin, and lipopolysaccharide. *Microcystis aeruginosa*, which is the most common toxin-producing cyanobacteria found worldwide, produces microcystins Many studies showed that these microcystins and nodularin inhibit in vitro activity of protein phosphatase in a cytosolic fraction of mouse liver.[12,13] Liver is reported as the target organ that shows the greatest degree of histopathological change when animals are poisoned by these cyclic peptides. The cause of death in mice is at least partially known and is concluded to be hypovolemic shock caused by interstitial hemorrhage.[14]

Extraction with supercritical fluids as solvents have received wide attention recently. A number of potential advantages, including more rapid extraction rates, more efficient extractions, increased selectivity, and potential for combined analyte fractionation, in conjunction with extraction, are possible with supercritical fluid extraction (SFE).

These advantages of SFE accrue from the properties of a solvent at temperatures and pressures above its critical point. At elevated pressure, this single phase will have properties which are intermediate between those of the gas and the liquid phases and are dependent upon the fluid composition, pressure, and temperature. The compressibility of supercritical fluids is large, just above the critical temperature, and small changes in pressure result in large changes in density of the fluid.[15] The density of a supercritical fluid is typically

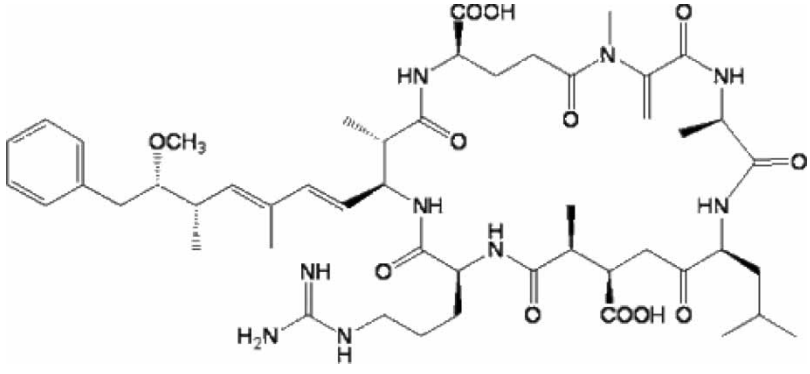


Figure 1. Structure of microcystin LR.

$10^2 - 10^3$ times that of the gas. Consequently, molecular interactions increase due to shorter intermolecular distances. However, the diffusion coefficient and viscosity of the fluid, although density dependent, remain more like those of a gas.[15] The 'liquid-like' behaviour of a supercritical fluid results in greatly enhanced solubilizing capabilities, compared to the corresponding liquid. These properties allow solvent strengths similar to liquids, but with greatly improved mass-transfer properties which provide the potential for more rapid extraction rates and more efficient extraction due to better penetration of the matrix.

Supercritical CO_2 extraction has been commercially applied to caffeine removal from green coffee beans[16] and extraction of PAHs in a roadside soil.[17] Other applications have been described in many articles.[18,19]

This study describes an investigation conducted to evaluate the applicability and efficiency of SFE methods for the extraction of blue-green algal hepatotoxin, microcystin LR from cyanobacteria. In our laboratory, in the past, aqueous acetic acid modified CO_2 was used for the extraction of microcystins from cyanobacteria,[20] and aqueous methanol was also used as a modifier.[21] In this study, we tried a new organic solvent, i.e., aqueous acetonitrile, as a co-solvent, so that we could obtain a higher extraction efficiency of the microcystin LR from cyanobacteria than with earlier experiments.[20,21]

EXPERIMENTAL

Reagents and Chemicals

Supercritical fluid extractions were performed with carbon dioxide, SFC grade (Scott Specialty Gases, Plumsteadville, PA). All solvents were HPLC grade from Aldrich (Milwaukee, USA). The microcystin LR standards were purchased from Wako (Japan).

SFE System

Cyanobacterial cells were collected in summer from lakes and rivers. Cells were collected with a plankton net, or surface scums were collected by a dip net, when dense surface scum was floating. Algal cells were freeze-dried for extraction. Dried cells (100 mg), pre-extracted with a ternary mixture (90% CO_2, 9.5% acetonitrile, 0.5% water) at 40°C and 250 atm, were spiked with 10 μg of microcystin LR and transferred to an extraction vessel. Supercritical fluid extractions of microcystins from cyanobacteria were performed using a Jasco (Tokyo, Japan) LC-900 SFE system. The schematic diagram of the system is shown in Figure 2. This system consisted of three sections: fluid delivery, extraction, and collection. The fluid delivery section included two pumps which delivered liquid carbon dioxide and a modifier solvent, separately. In the extraction section, supercritical fluid extractions were performed

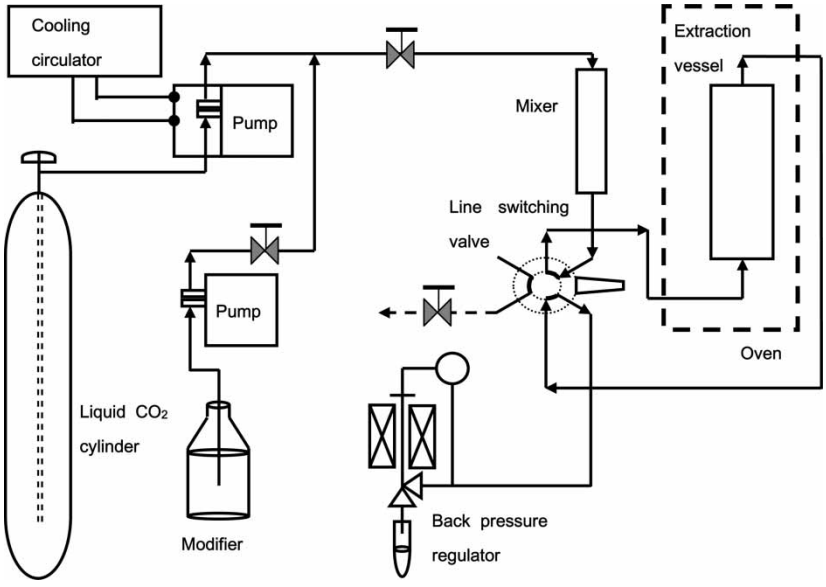


Figure 2. Schemetic flow diagram of SFE modular system.

with carbon dioxide modified aqueous acetonitrile. The collection section included a back-pressure regulator, which kept the pressure of the extraction vessel at a desired value.[22] The effluent flowing through the back-pressure regulator reduced its pressure to atmospheric pressure and, thereby, solutes in the effluent reduced their solubility to virtually zero. In this way, the solutes were deposited and collected in a collection vessel. Since we used aqueous acetonitrile modified CO_2 as a extracting solvent, the extracts were collected in a liquid solvent in the collection vessel. The detailed list of components of the system are given in the Figure 2 caption.

HPLC System

The determination of the microcystin LR was performed with a Beckmann 116 pump (System Gold Programmable Solvent Module 126), a 10 mm × 15 cm ODS column and a Hewlett Packard HPLC 1100 series diode array detector coupled in series; [methanol:acetonitrile = 1:1]:0.025 M phosphate buffer (pH: 2.9) = 44:56 was used as a mobile phase, at a flow rate of 1.0 mL/min.

RESULTS AND DISCUSSION

The main limitation of the most often used supercritical fluid, CO_2, is its limited ability to dissolve polar molecules, even at very high densities.

Other possible neat fluids for such purposes often are reactive, flammable, or toxic. Alternatively, the characteristics of the supercritical fluid mobile phase can be varied by the addition of miscible compounds to the supercritical CO_2. The microcytin LR included in this study is sparsely soluble in neat CO_2. As mentioned earlier, we have already tried to extract microcystin LR from cyanobacteria using CO_2 + aqueous acetic acid[20] and CO_2 + aqueous methanol.[21] One of the difficulies with using aqueous acetic acid as a co-solvent was the high fluctuation of the pressure gauge which results from the clogging of the extraction line. The reason we used aqueous acetonitrile as a co-solvent in this study is that acetonitrile is a little more polar than methanol, although acetonitrile has similar physicochemical properties to methanol. The dielectric constant of acetonitrile at 20°C is 37.5 (in case of methanol, 32.7) and the polarity index of acetonitrile is 5.8 (in case of methanol, 5.1)[23] The use of acetonitrile was expected to give a higher extraction efficiency for the extraction of the polar compound, microcystin LR, from cyanobacteria, than methanol. As expected, with aqueous acetonitrile-modified CO_2, 98% of the microcystin LR was extracted, whereas, with aqueous methanol-modified CO_2, 95% of the microcystin LR was extracted.[21] However, when neat CO_2 was used as the extraction fluid, at 40°C and 250atm, no microcystin LR could be extracted from freeze-dried cyanobacterial cells.

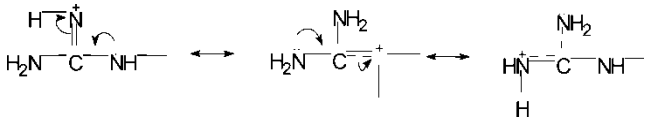
In supercritical fluid extraction, the extraction of an analyte depends on its distribution between the supercritical fluid and the sorptive sites in the sample matrix. In general, for predicting optimal extraction conditions, one must have two considerations in mind: the ability of the supercritical fluid to compete with the analytes for the sorptive sites, and the solubility of the analytes in the supercritical fluid. The latter usually appears to be more important. From Figure 1, microcystin LR contains a strongly basic functional group (NHCNH-NH_2), i.e., a guanidine moiety. This microcystin LR would be

Table 1. Supercritical fluid extraction of microcystin LR using various temperatures

Temperature, °C	% extraction of microcystin LR
70	27 ± 3
46	33 ± 2
80	87 ± 2
90	98 ± 2

Experimetal condition: 75 min extraction at 250 atm, CO_2 flow 2.0 ml/min and modifier flow 0.2 ml/min. RSDs based on triplicate extractions under each condition.

present mainly in the following cationic form:



The poor extraction result with neat CO_2 is probably caused by the fact that microcystin LR consists of fairly polar functional groups. The use of co-solvents can have a profound effect on increasing the solubility levels of polar solutes in supercritical fluids. In this study, our experimental results have indicated that 95% aqueous acetonitrile (95% acetonitrile + 5% water) wase the most suitable co-solvent for the supercritical fluid extraction of

Table 2. Supercritical fluid extraction of microcystin LR using various pressures

Pressure, atm	% extraction of microcystin LR
280	61 ± 4
270	82 ± 3
260	94 ± 3
250	98 ± 3
240	82 ± 3

Experimetal condition: 75 min extraction at 40°C, CO_2 flow 2.0 ml/min and modifier flow 0.2 ml/min. RSDs based on triplicate extractions under each condition.

Table 3. Supercritical fluid extraction of microcystin LR using different compositions of aqueous acetonitrile as a co-solvent

Fluid phase	% extracion of microcystin LR
90% CO_2 + 10% acetonitrile	27 ± 2
90% CO_2 + 9.5% acetonitrile + 0.5% water	98 ± 2
90% CO_2 + 9.0% acetonitrile + 1.0% water	83 ± 2
90% CO_2 + 8.5% acetonitrile + 1.5% water	53 ± 3
90% CO_2 + 8.0% acetonitrile + 2.0% water	12 ± 2

Experimetal condition: 75 min extraction at 40°C and 250 atm, CO_2 flow 2.0 ml/min and modifier flow 0.2 ml/min. RSDs based on triplicate extractions under each condition.

microcystin LR from dried cyanobacterial cells. The co-solvent flow rate was 0.2 mL/min and the supercritical CO_2 fluid flow rate was 2.0 mL/min. Therefore, the most suitable medium for the supercritical fluid extraction of microcystins was a ternary mixed fluid (90% CO_2, 9.5% acetonitrile, and 0.5% water). When 95% aqueous acetonitrile was used as a co-solvent at 40°C and 250atm, 98% of microcystin LR was extracted. It is important to find the optimum SFE operating conditions which would result in the most efficient extraction of microcystin LR from cyanobaterial cells. In particular, the pressure and temperature of the supercritical fluid, which are the two most important parameters to be optimized, for the most SFE experiments. To find the optimum extraction temperature, the temperature of the extraction vessel was varied from 40°C to 70°C (Table 1). The best extraction efficiency was shown at 40°C; therefore the temperature used in this study was 40°C. The solvating strength of a supercritical fluid is related to its density, a parameter which is primarily dependent upon pressure. For this study, the

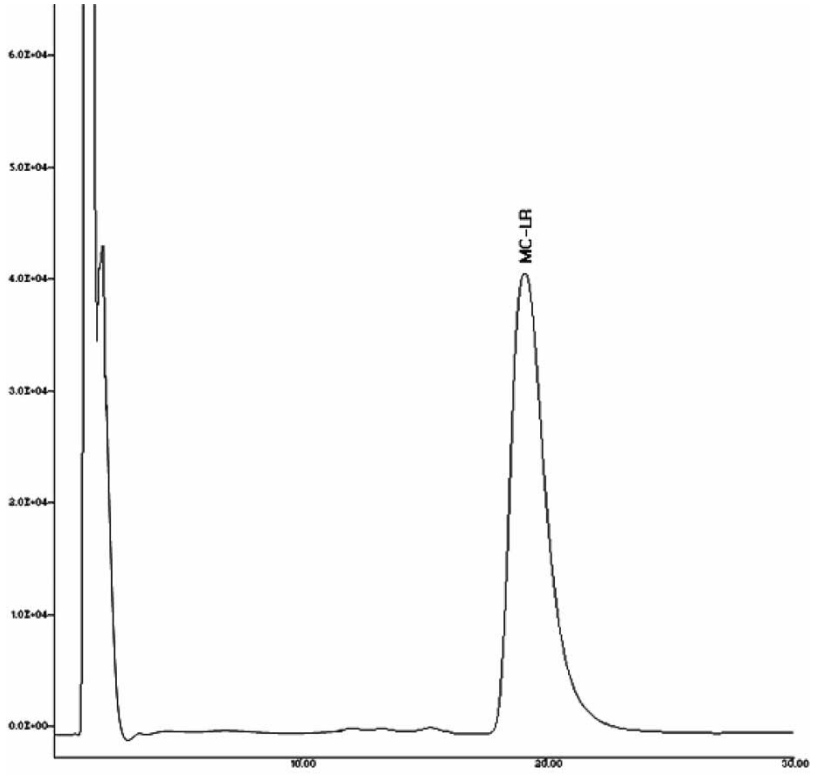


Figure 3. High performance liquid chromatogram of standard microcystin LR. HPLC condition: [methanol: acetonitrile = 1:1]: 0.025 M phosphate buffer (pH: 2.9) = 44 : 56, 1.0 mL/min, 238 nm.

extraction pressure was increased from 240 atm to 280 atm at intervals of 10 atm (Table 2). The extraction efficiency increases with increasing pressure of extracting fluid until the pressure reaches 250 atm. At higher pressures than 250 atm, the extraction efficiency decreases. Other compositions of acetonitrile and water were also tried, to find the best extraction condition (Table 3).

A very interesting finding in these experiments was the effect of water on the extraction of microcystin LR from cyanobacteria. From Table 3, it can be seen that, when pure acetonitrile was used as a co-solvent, only 27% of the microcystin LR was extracted. A poor extraction efficiency with no water indicates that water plays an important role in the supercritical fluid extraction of microcystins. Water has a much greater dielectric constant than acetonitrile (water; 80.1, acetonitrile; 37.5).[23] When water is added, two functional groups of opposite charge in the microcystin LR can be separated easily. Microcystin LR has a basic functional group ($NHCNH\text{-}N^{+}H_2$) and an acidic functional group (COO^{-}). The work required to separate these two functional groups is rapidly decreased by adding water, a co-solvent with a high

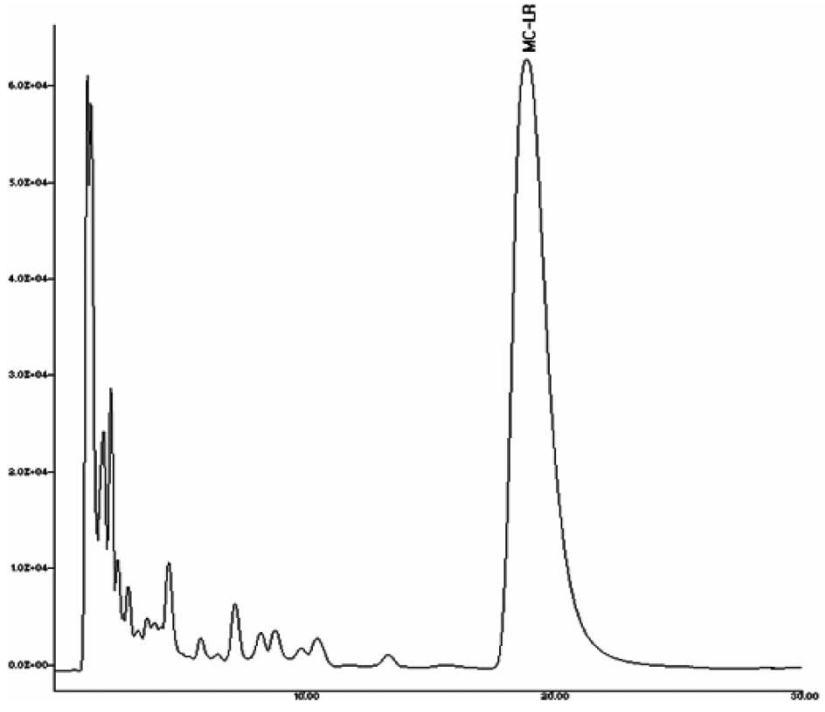


Figure 4. High performance liquid chromatogram of extracted microcystin LR using SFE (with ternary mixed fluid (90% CO_2, 9.5% acetonitrile, 0.5% water) at 40°C and 250 atm. HPLC condition: [methanol: acetonitrile = 1:1]: 0.025 M phosphate buffer (pH: 2.9) = 44:56, 1.0 mL/min, 238 nm.

dielectric constant. The extent of separation of two functional groups of opposite charge in the microcystins can affect the solubility of microcystins in methanol modified supercritical fluid CO_2.

From Figures 3 and 4, it is noted that, when the samples are extracted by SFE, more small compounds are also extracted. However, since these small compounds elute earlier than microcystin LR, the peaks of those compounds do not overlap with the peak of microcystin LR in the HPLC chromatogram. The SFE procedure has a unique advantage over the SPE procedure in terms of analysis time; the former takes about 95 min, which includes 75 min extraction time and 20 min modifier evaporating time, and the latter takes about 8 hours.

In conclusion, the microcystin LR was successfully extracted with a ternary mixture (90% CO_2, 9.5% acetonitrile, 0.5% water) at 40°C and 250 atm. Sample handling steps are minimized, thus reducing the possible losses of analytes and saving analysis time. No clean-up steps are employed since the SFE with aqueous acetonitrile modified CO_2 gives clean extracts which can be directly analyzed with HPLC. The polar carbon dioxide-aqueous acetonitrile fluid system gave a higher extraction efficiency than any other mixed fluid systems for the extraction of the polar microcystin LR from cyanobacteria.

ACKNOWLEDGMENT

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